REMARKS

Claims 1, 6-8, and 11 are pending in the application. Claim 1 has been amended. Claims 2-5 and 9-10 have been cancelled in this or a previous amendment. Applicants expressly reserve the right to pursue the subject matter of cancelled claims in this or a subsequent application. Support for the amendment to claim 1 can be found in original claim 8 and throughout the examples. No new matter has been added by virtue of this amendment.

Claim 11 was rejected under 35 U.S.C. §112, first paragraph, allegedly because the specification, while being enabling for the treatment of diseases listed in claim 11, does not reasonably provide enablement for prevention of such diseases.

The rejection is traversed.

The use of retinoic acid for the treatment and prevention of cancer is well known in the art. See, for example, the background of the instant application and the enclosed manuscript by Y. Choi, et al., for a discussion of the use of retinoic in the treatment and prevention of cancer.

In contrast, the present invention provides a novel retinoic acid controlled release formulation suitable for the administration of retinoic acid to patients. Moreover, claim 11 provides pharmaceutical compositions for the treatment or prevention of various cancers wherein the pharmaceutical composition comprises the controlled drug releasing system of the invention and a pharmaceutically acceptable carrier. Thus, the specification provides ample enablement for the drug delivery system comprising a therapeutic agent which is know to have an effect in the treatment or prevention of various cancers.

Thus, claim 11 is fully compliant with the requirements of 35 U.S.C. §112, including the requirements of §112, first paragraph.

Claims 1 and 6-8 were rejected under 35 U.S.C. §112, second paragraph, as being allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 1 as amended provides a controlled drug release system for retinoic acid comprising a block copolymer of a biodegradable polymer and an amphiphilic polymer with a mixing ratio of between 1:1-20%. Thus, claim 1 is fully compliant with the requirements of 35 U.S.C. §112, including the requirements of §112, second paragraph.

Claims 1 and 6-8 were rejected under 35 U.S.C. §103(a) as being allegedly obvious over Gref et al. (U.S. Patent 5,543,158) in view of Rodgers (U.S. Patent 5,534,261).

The rejection is respectfully traversed.

As the reference is understood, Gref teaches a microsphere formed of a block copolymer which consists of poly(alkylene oxide) blocks and poly(lactic acid-co-glycolic acid) blocks. The Gref microspheres further comprise a series of polyalkylene glycol chains on the surface of the microsphere. However, Gref neither discloses nor suggests microspheres composed of a mixture of a biodegradable copolymer and an AB type di-block copolymer. Further, this reference does not explicitly disclose the application of retinoic acid to the microsphere.

In contrast, the present invention provides microsphere compositions in which the biodegradable polymer and the block copolymer are mixed together such that these microsphere compositions can be sterilized by ethylene oxide gas. The block copolymer component of the

microsphere composition increases the crystallinity of the microsphere thereby preventing deformation of the microsphere during ethylene oxide sterilization procedures.

In contrast, conventional microspheres, such as those taught by Gref, which are prepared from a single biodegradable block copolymer, are stuck together after the sterilization by ethylene oxide gas, and so the conventional microspheres cannot be sterilized by ethylene oxide gas but can be by only γ-ray irradiation.

Rodgers fails to overcome the limitations of the Gref disclosure. As the reference is understood, Rodgers discloses microspheres comprising all trans retinoic acid and a biodegradable polymer such as poly(dl-lactides), poly(dl-lactide-co-glycolides), or polycaprolactones. However, Rodgers never discloses or suggests combining an AB type di-block copolymer with the biodegradable polymer as claimed in the present invention.

Neither Gref, Rodgers or any combination thereof teach the microspheres of the present invention. More particularly, the present invention would not have been obvious to one skilled in the art would not have been motivated by any combination of the cited references. Thus the drug delivery system for retinoic acid comprising a microsphere comprising a mixture of a block copolymer and a biodegradable polymer where the microsphere has a retinoic acid dispersed therein would not have been obvious to one skilled in the art based on Gref and Rodgers.

Claims 1, 6-8, and 11 were rejected under 35 U.S.C. §103(a) as being allegedly unpatentable over Cha (WO 97/15287) in combination with Rodgers and in further combination with Lippman (1992).

The PCT publication of Cha et al. ('287) discloses an aqueous mixture of a biodegradable block polymer and a peptide/protein for delivery to a patient. The aqueous mixture is injected into a patient and the block polymer, which forms a hydrogel at body temperature, thereby trapping the peptide or protein in the gel matrix. The invention of Cha ('287) neither discloses nor suggests a microsphere in which the biodegradable copolymer is combined with AB type di-block copolymer, and further with retinoic acid as claimed in the present invention.

The disclosures of Rodgers and Lippman fail to overcome the limitations of Cha ('287). Thus no combination of Cha ('287), Rodgers and Lippman teach or disclose the drug delivery systems of the invention comprising one or more retinoic acid dispersed in a microparticle composed of a biodegradable copolymer and an AB type di-block copolymer

Thus claim 1 is patentable over the combined teachings of Cha ('287) in view of Rodgers and Lippman. Claims 2-8 and 11 depend from claim 1 and are therefore also patentable over Cha ('287) in view of Rodgers and Lippman.

Claims 1, 6-8, and 11 were rejected under 35 U.S.C. §103(a) as being allegedly unpatentable over Cha (U.S. Patent 5,665,428) in combination with Rodgers and in further combination with Lippman (1992).

The rejection is traversed.

As the reference is understood, Cha ('428) teaches a microparticle comprising a polypeptide dispersed in a block copolymer capable of forming a hydrogel after injection into a patient or an aqueous media where a polypeptide is released from the hydrogel after injection. Cha ('428) neither discloses nor suggests any microparticle composition

comprising a biodegradable polymer and an AB type block copolymer. Moreover Cha ('428) neither discloses nor suggests any microparticle compositions comprising a biodegradable polymer and an AB type block copolymer having at least one retinoic acid dispersed therein.

Rodgers fails to overcome the limitations of Cha ('428). Rodgers, as discussed *supra*, neither discloses nor suggests combining the AB type di-block copolymer with the biodegradable polymer as claimed in the present invention. Thus, no combination of Cha ('428) and Rodgers teach or suggest to one of ordinary skill in the art a drug delivery system comprising retinoic acid dispersed in a mixture of an AB type di-block copolymer with the biodegradable polymer as provided by claim 1 of the present invention.

The disclosure of Lippman et al., fails to overcome the limitations of Cha ('428) in view of Rodgers. As the reference is understood, Lippman teaches the delivery of 13-cis-retinoic acid and interferon α-2a for the treatment of cervical cancer. Lippman neither discloses nor suggests drug delivery systems for the delivery of retinoic acid comprising microspheres composed of a mixture of AB type di-block copolymer and biodegradable polymer. Thus Lippman fails to overcome the limitations of the combined teachings of Cha ('428) and Rodgers.

Thus claim 1 is patentable over the combined teachings of Cha ('428) in view of Rodgers and Lippman. Claims 2-8 and 11 depend from claim 1 and are therefore also patentable over Cha ('428) in view of Rodgers and Lippman.

The claims, as amended, provide controlled drug release systems for retinoic acid and pharmaceutical compositions comprising same. More particularly, the controlled drug release systems provided by the invention are characterized in that retinoic acid is incorporated into a microsphere prepared by mixing a biodegradable polymer, which is selected from the group

consisting of poly-*L*-lactic acid, poly-*D,L*-lactic acid and poly(lactic-co-glycolic acid), and an amphiphilic AB type di-block copolymer, which is poly-*L*-lactic acid-polyethyleneglycol or poly(lactic acid-co-glycolic acid)-polyethyleneglycol, together, wherein the retinoic acid is selected from the group consisting of all-trans-retinoic acid, 13-cis-retinoic acid, 9-cis-retinoic acid, other retinoids and the mixture thereof, and the mixing ratio of the biodegradable polymer and the amphiphilic block copolymer is 1:1-20% by weight.

None of the references cited by the Examiner, taken alone or in combination, disclose or suggest the claimed invention. Thus, the rejections of the claims under §103 should be withdrawn.

In view thereof, reconsideration and withdrawal of the rejections is requested.

It is believed the application is in a condition for immediate allowance.

Respectfully submitted,

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John B. Alexander (Reg. No.: 48,399)
Dike, Bronstein, Roberts & Cushman
Intellectual Property Group
EDWARDS & ANGELL, LLP
P.O. Box 9169
Boston, MA 02209
Tel. (617) 439-4444

Chemopreventive efficacy of all-*trans*-retinoic acid in biodegradable microspheres against epithelial cancers: Results in a 4-nitroquinoline 1-oxide-induced oral carcinogenesis model

Yongdoo Choi[1], Sang Yoon Kim[2,*], Kyeongsoon Park[1], Jehoon Yang[2], Kyung-Ja Cho[3], Hyun Ja Kwon[2], and Youngro Byun[1,**]

[1]Department of Materials Science and Engineering, Kwangju Institute of Science and Technology, Gwangju 500-712, Korea

[2]Department of Otolaryngology, Asan Medical Center, College of Medicine, University of Ulsan, Seoul 138-736, Korea

[3]Department of Pathology, Asan Medical Center, College of Medicine, University of Ulsan, Seoul 138-736, Korea

** Corresponding author: Youngro Byun, Ph.D.
Department of Materials Science and Engineering,
Kwangju Institute of Science and Technology,
1 Oryong-dong, Puk-gu, Gwangju 500-712, Korea
Tel) +82-62-970-2312, Fax) +82-62-970-2304
E-mail: yrbyun@kjist.ac.kr

* Co-corresponding author: Sang Yoon Kim, M.D.
Department of Otolaryngology, Asan Medical Center
College of Medicine, University of Ulsan

388-1 Poongnap-dong, Songpa-gu, Seoul 138-736, Korea

Tel) +82-2-3010-3715, Fax) +82-2-489-2773

E-mail: sykim2@amc.seoul.kr

ABSTRACT

Purpose. In order to overcome "acute retinoid resistance' of all-trans-retinoic acid (atRA) in oral delivery, parenteral administration of atRA-loaded biodegradable microspheres was suggested in our previous study. In this study, chemopreventive efficacy of atRA-loaded microspheres on epithelial cancers was evaluated by using the model of 4-nitroquinoline 1-oxide-induced oral carcinogenesis.

Methods. Ninety-eight F344 rats (male) were divided into four groups. Rats in the groups 1 to 3 were given 20 ppm of 4-nitroquinoline 1-oxide (4-NQO) in drinking water for 8 weeks and treated with placebo or atRA-loaded microspheres. The group 4 was put on a basal diet and tap water without 4-NQO and it served as an untreated control. The histology of the hard palate and tongue was evaluated after 22 weeks.

Results. When 50 mg atRA/kg of atRA-loaded microspheres were subcutaneously administered once in rats, the atRA concentration in plasma was found to be around 6.5 ng/ml for 7 weeks and only minimal signs of toxicity were observed. A single injection of atRA-loaded microspheres significantly suppressed oral carcinogenesis; however, additional injections of atRA-loaded microspheres did not show any further reduction in carcinogenesis.

Conclusions. The long-term treatment of atRA by using biodegradable microspheres has been found to suppress oral carcinogenesis and this new drug delivery system would be an effective method of using atRA as a chemopreventive agent.

KEY WORDS: Chemoprevention, oral carcinogenesis, all-trans retinoic acid, microspheres, PDLLA

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is an aggressive epithelial malignancy that is now the sixth most prevalent neoplasm. In spite of much advancement in treatment modality, the long-term survival rate of HNSCC has remained less than 50% for the last 30 years. Of the hurdles that prevent its long-term survival, the frequent development of second primary cancers had posed as the major threat (1-3). Among the many chemotherapeutic agents, 13-*cis*-retinoic acid have been effective in reversing premalignant oral lesions and preventing the second primary cancers (3).

All-*trans*-retinoic acid (atRA), an active isomer of 13-*cis*-retinoic acid, plays an essential role in the regulation of cell differentiation and proliferation in epithelial tissues (4), and the effects of retinoic acid on HNSCC have been proved *in vitro* and *in vivo* (5, 6). Presently, however, the clinical application of atRA is strictly limited due to both the "acute retinoid resistance" and toxicity (7, 8). Pharmacokinetic studies have demonstrated that when atRA was orally administered on a chronic daily basis, the area under the curve (AUC) and the half-life of atRA in plasma rapidly decreased at every repeated dose, recording a very short half-life of less than one hour (7). This observation has been attributed to accelerated metabolism brought on by a specific cytochrome P450, which is induced by atRA in the liver (9, 10). Consequently, the atRA concentration in plasma cannot be maintained in the therapeutic range in long-term treatments.

In order to overcome the difficulties faced in the clinical application of atRA, particularly its rapid first-pass metabolism, several drug delivery systems such as microemulsions, liposomes, and nanoparticles have been investigated (11-14). Parenteral administration of the atRA-encapsulated liposomes has shown both decreased metabolism of atRA in the liver and reduced toxicity. Besides the drug delivery system, the method of combined therapy has been developed

using atRA with metabolism inhibitors such as liarozole, fluconazole and ketoconazole (15). New retinoids that selectively bind and activate only one subtype of nuclear retinoid receptor have also been studied (16).

In this study, atRA-loaded biodegradable microspheres have been found to maintain the plasma concentration of atRA in the therapeutic range for a long period. When atRA was released from the subcutaneously injected microspheres, the first-pass metabolism was avoided, and the continuously released atRA could maintain its plasma concentration in the therapeutic range for a long period. The preventive efficacy of atRA-loaded microspheres was evaluated in a 4-nitroquinoline 1-oxide-induced oral carcinogenesis model. Microspheres containing atRA were prepared with poly(D,L-lactide) (PDLLA) as a matrix polymer and poly(L-lactide)-poly(ethylene glycol) (PLE) block copolymers were blended in the matrix at 8 wt% to control the release rate of atRA.

MATERIALS AND METHODS

Materials

All-*trans*-retinoic acid and Lugol's solution were obtained from Sigma Chemical Co. (St. Louis, MO). Poly(D,L-lactide) (PDLLA, Res R202, M_w 17,500) was purchased from Boehringer Ingelheim Co. (Ingelheim, Germany). Monomethoxy polyethylene glycol (mPEG, M_n 5,000), L-lactide, poly(vinyl alcohol) (PVA, 98% hydrolyzed, M_w 13,000-23,000), 4-nitroquinoline 1-oxide (4-NQO), ammonium acetate, potassium hydrogen phosphate and isobutyl alcohol (HPLC grade) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Acetonitrile and glacial acetic acid were of HPLC grade, and were supplied by Mallinckrodt Baker Inc. (Phillipsburg, NJ).

Preparation of atRA-loaded microspheres

Poly(L-lactide)-poly(ethylene glycol) diblock copolymer (PLE) was synthesized with mono-methoxy PEG (mPEG, M_n 5,000) and L-lactide by solution polymerization as described in a previous study (17). The number average molecular weight (M_n) and molecular weight distribution of the synthesized PLE were 32,500 Dalton and 1.46, respectively. PDLLA/PLE microspheres containing atRA were prepared using a solvent evaporation method in oil-in-water emulsion. PDLLA (4 g), atRA (0 wt% for placebo microspheres and 10 wt% for atRA-loaded microspheres) and PLE (8 wt%) were concurrently dissolved in dichloromethane. The mixture was poured into 600 ml of an aqueous solution containing 2% (w/v) of PVA, while mixing vigorously using a mechanical stirrer (IKA LABORTECHNIK, Selangor, Malaysia) at 1,000 rpm. After mechanical stirring for 10 min, the resulting suspension was gently stirred for 3 h at 40℃ with a magnetic stirrer to evaporate dichloromethane. The microspheres were separated by centrifugation at 12,000 rpm for 10 min. Thus obtained microspheres were washed with distilled water, centrifuged four times and freeze-dried.

The prepared microspheres were sterilized by gamma-irradiation before injecting into rats. Gamma-irradiation sterilization was performed in open air at room temperature in a ^{60}Co source with a total dose of 25 kGy, and the sterilized microspheres were stored at –20℃ before use.

Release study of atRA from the microsphere in vivo

Six F344 rats, 6 weeks old and purchased from Japan SLC Inc. (Tokyo, Japan), were used for measuring the amount of atRA released from the subcutaneously administered microspheres. After a dose of 50 mg atRA/kg of atRA-loaded microspheres had been

administered subcutaneously to rats, blood was sampled at a given time interval and assayed by high performance liquid chromatography (HPLC) as described by Buggé *et al* (18). A Hitachi HPLC system (D-7000 series Hitachi Ltd., Tokyo, Japan) with TSK-gel ODS-80T_M column (4.6 x 250 mm, Tosoh Co., Tokyo, Japan) was used. Briefly, 150 µl of a 1:1 mixture of acetonitrile and isobutyl alcohol was added to 200 µl of plasma and vortexed for 1 min. After the addition of 120 µl of saturated K_2HPO_4 solution and mixing for 30 sec, the samples were centrifuged for 2 min. The organic upper layer (80 µl) was analyzed by HPLC. A linear gradient from initial condition of 100% phase A to 100% phase B was adopted over 15 min. The final condition was maintained for 15 min, prior to a 5 min re-equilibration to the initial condition. The mobile phase A consisted of a mixture of water, acetonitrile and acetic acid in the ratio of 100:100:1, whereas B consisted of water, acetonitrile and acetic acid in 190:10:0.08 ratio. Both the mobile phases contained 10 mM of ammonium acetate. The atRA concentration was measured by absorbance at 365 nm.

Experimental procedure of carcinogenesis

Male F344 rats were housed in a holding room kept under the following controlled conditions: 23±3℃ temperature, 50±10% humidity, and 12 h light/dark cycle. After 2 weeks of quarantine, healthy rats were randomized into four groups. A 4-NQO solution was prepared at the concentration of 20 ppm in tap water and stored in dark at 4℃ until used (19). Dark amber bottles were used for the 4-NQO solution to protect it from decomposition by light.

Ninety-eight rats were divided into four groups as shown in Figure 1. Rats of the groups 1 to 3 were freely given 20 ppm 4-NQO in drinking water for 8 weeks to induce oral carcinogenesis. Placebo microspheres without any atRA were administered subcutaneously to rats

in the group 1. For the groups 2 and 3, a dose of 50 mg atRA/kg of atRA-loaded microspheres were injected subcutaneously when the feeding of 4-NQO was started. The sterilized atRA-loaded microspheres were dispersed in a sterile, non-pyrogenic normal saline solution at the concentration of 0.5 g/ml without the addition of any surfactants, and were subcutaneously injected by a sterilized disposable syringe with a 20-gauge needle. For the group 3, the same dosage of atRA-loaded microspheres was subcutaneously administered at every 8 weeks interval. The group 4 was given the basal diet and tap water without 4-NQO, and it served as an untreated control. The dosage of atRA-loaded microspheres was determined according to our previous study on the subacute toxicity (20).

All rats were carefully observed daily and their body weights were measured weekly during the experiment period of 22 weeks. At necropsy, hard palates and tongues of each rat were collected for histological analysis. During the collection of the tissues, the size of the projected tumors on tongue mucosa was measured using the equation of width × length × height × $\pi/6$ (21). The mucosa of palate and tongue were brushed over with a 2% Lugol's dye solution to detect dysplasia and carcinoma in mucosal layer (22). Suspicious areas of dysplasia and carcinoma appeared white (i.e., unstained) or yellow. Each hard palate and tongue was sliced into three pieces passing through the suspicious areas as shown in Figure 2. The sliced tissues were fixed in a 10% buffered formaldehyde solution, embedded in paraffin, sectioned with a microtome, and stained with hematoxyline and eosin for histological examination. The most advanced stage of carcinogenesis as viewed from various histological pictures of the hard palate and tongue in a rat was taken as the stage of carcinogenesis for that rat. Epithelial lesions (hyperplasia, dysplasia, and neoplasm) in the oral cavity were diagnosed according to the criteria described by Bánóczy and Csiba (23). Basal layer was thickened, and the loss of polarity was started in rats with hyperplasia. And, the loss of polarity of epithelium was found throughout the whole thickness in

rats with dysplasia. Invasion into dermis, well-differentiated SCC in dermis, and keratin pearls were observed in rats with invasive SCC.

Statistical analysis

Preventive effects of the microspheres against development of lesions were statistically evaluated using the Wilcoxon rank sum test. Other data were expressed as mean ± SD values, and statistical analyses of the data were performed with the use of the Student's t-test.

RESULTS AND DISCUSSION

The atRA-loaded microsphere showed a smooth and dense surface structure, and its size ranged from 20 to 100 μm. The optimum loading contents of PLE and atRA in PDLLA microsphere were 8 wt% and 10 wt%, respectively. The sterilized microspheres were analyzed by gel permeation chromatography (Waters LC system coupled to a Waters 410 Differential Refractometer, Waters Co., Milford, MA), ^{1}H-NMR spectrum (JEOL JNM-LA 300 WB, 300 FT-NMR, Tokyo, Japan) and HPLC (Hitachi HPLC system, D-7000 series, Hitachi Ltd., Tokyo, Japan). The molecular weight of PDLLA was decreased by about 850 Dalton (~ 5.3 % decrease) during the sterilization since PDLLA polymer chain could be randomly cleaved by gamma irradiation. The chemical structures of atRA, however, appeared intact after gamma-irradiation sterilization.

In an oral route, atRA is rapidly metabolized in the liver, leading to a very short half-life of less than one hour (7). Therefore, parenteral administration of atRA-loaded microspheres was suggested in this study to enhance the clinical efficacy of atRA for chemoprevention. ATRA in

the biodegradable microsphere was slowly released and the atRA concentration in the plasma of F344 rats could be maintained greater than 3 times of the physiological level for 7 weeks. When atRA-loaded microspheres were subcutaneously administered to rats at the dose of 50 mg atRA/kg, the atRA concentration in plasma reached 18.5 ± 6.3 ng/ml at one week, as shown in Figure 3. Afterwards, the atRA concentration in plasma was reduced gradually and maintained in the range of 9.3 to 3.2 ng/ml until the 7th week with the mean value of around 6.5 ng/ml. After 7 weeks, the concentration of atRA in plasma decreased below the detection limit (3 ng/ml). When the same dose of placebo microspheres was administered, atRA was not detected by HPLC, which means that the plasma concentration of atRA was maintained below 3 ng/ml under the fed condition. The endogenous level of atRA in the plasma was reported to be very low (mean value: ~ 1.5 ng/ml), being in the range of 0.5 ~ 2.7 ng/ml in rats (24, 25), and 0.92 ~ 3.54 ng/ml in human (26). In this study, the plasma level of atRA was not elevated more than 3 ng/ml under the fed condition. Although the minimum effective plasma concentration of atRA needed to prevent carcinogenesis has not been reported, several *in vitro* cell studies have indicated that there is a dose- and time-dependant transcriptional activation of nuclear retinoic acid receptors (RARs: RAR-α, -β, and -γ) above the 3 ng/ml atRA concentration (27, 28).

For the carcinogenesis experiments, the dose of atRA-loaded microspheres (50 mg atRA/kg) was determined from a previous subacute toxicological study, and the injection interval of the microspheres was decided as 8 weeks since the plasma concentration of atRA was maintained above 3 ng/ml for 7 weeks. Repeated injections of atRA-loaded microspheres were highly tolerable, and minor signs of toxicity such as decrease in activity and body weight gain of the rats in the atRA-treated groups had been also observed transiently after 2 weeks of microsphere injection. The state of the rats, however, was gradually improved with time and no

adverse tissue reactions at the injection sites of atRA-loaded microspheres were observed at this dose for about 25 weeks (20, 29).

Rats in the groups 2 and 3 showed a temporary decrease both in their activity and in body weight gain; gradually, there was improvement in their activity and body weight gain after 2 weeks. Ultimately, body weights of rats were not different statistically between each group at 22 weeks (Figure 4). However, the rats, which developed large tumors, showed a significant decrease in their body weights after tumor had projected in tongue. Projected tumors were observed in tongues and not in hard palates (Figure 5(a)). In the group 1, a projected tumor lager than 10 mm^3 was observed in the tongue of 7 rats (21.9%); in 4 of these 7 rats, tumor was larger than 100 mm^3. For the group 2 and 3, the size of the projected tumors was greater than 10 mm^3 and the incidence rates were 13.3% and 7.1%, respectively (Figure 5(b)).

Histological evaluations for carcinogenesis were performed for hard palates and tongues (Figure 6). Basal layer was thickened, and the loss of polarity was started in rats with hyperplasia. And, the loss of polarity of epithelium was found throughout the whole thickness in rats with dysplasia. Invasion into dermis, well-differentiated SCC in dermis, and keratin pearls were observed in rats with invasive SCC. The development of preneoplasm and neoplasm in both sites are shown in Tables I and II. In hard palates of the group 1, most of rats showed that carcinogenesis proceeded up to dysplasia (40.6%) or invasive SCC (43.8%). On the other hand, in the group 2 that had been administered atRA-loaded microspheres once, 73.3% of the rats showed dysplasia and only 6.7% of the rats showed incidences of invasive SCC. In the group 3 that had been administered atRA-loaded microspheres repeatedly, carcinogenesis was further reduced to 64.3% dysplasia, 25% hyperplasia, and one of 28 rats showed no incidence of carcinogenesis. Therefore, carcinogenesis was significantly inhibited in the group 2 ($P < 0.05$ from the Wilcoxon rank sum test) and group 3 ($P < 0.01$ from the Wilcoxon rank sum test) as

compared to group 1. However, the incidence of invasive SCC could not be further reduced by repeated administration of atRA-loaded microspheres. The chemopreventive effect of atRA-loaded microspheres was also observed in the tongue (Table II); there was no significant difference in the chemopreventive effect between the groups 2 and 3 as it was in the hard palate. This study demonstrated that a subcutaneous administration of atRA-loaded microspheres maintained the plasma levels of atRA continuously above 3 ng/ml, thereby effectively suppressing the progress of oral carcinogenesis. As shown in tables, the higher frequency of dysplasia was observed in the atRA-treated groups with more frequent reduction of invasive cancer, suggesting that the progression of carcinogenesis was effectively arrested. The chemoprevention effect, however, was not further improved by additional injections of the atRA-loaded microspheres in spite of the fact that the formation of large tumors had somewhat decreased compared to when a single injection was given. It means that the dose regimen of 50 mg atRA/kg for the additional treatment was not enough to prevent further carcinogenic progression into invasive SCC. For further study, increased dose regimen for the additional treatment and concurrent administration of anti-inflammatory drug will be studied to enhance the efficacy of this delivery system. It has been reported that the rapid recovery from the intoxication by atRA could be achieved by combinations of anti-inflammatory drugs (30).

CONCLUSION

The problem of rapid decrease in the plasma concentration of atRA could be solved by the subcutaneous administration of atRA-loaded microspheres. ATRA released from the microspheres could avoid catabolism in the liver as well as maintaining the atRA concentration in the plasma in the therapeutic range for a long period. Therefore, atRA released from the

microspheres effectively reduced oral carcinogenesis, and treatment regimen was highly tolerable. Therefore, this new drug delivery system could serve as an effective method in using atRA as a chemopreventive agent.

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ABBREVIATIONS: atRA, all-trans-retinoic acid; 4-NQO, 4-nitroquinoline 1-oxide; HNSCC, Head and neck squamous cell carcinoma; AUC, area under the curve; PDLLA, poly(D,L-lactide); PLE, poly(L-lactide)-poly(ethylene glycol); mPEG, monomethoxy polyethylene glycol; PVA, poly(vinyl alcohol); invasive SCC, invasive squamous cell carcinoma;

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Table 1. Incidence of neoplasm on hard palate in F344 rats

Group	No. of rats examined	No. of rats (%)			
		Normal	Hyperplasia	Dysplasia	Invasive SCC
1	32	0/32 (0.0)	5/32 (15.6)	13/32 (40.6)	14/32 (43.8)
2	30	0/30 (0.0)	6/30 (20.0)	22/30 (73.3)	2/30 (6.7)[a]
3	28	1/28 (3.6)	7/28 (25.0)	18/28 (64.3)	2/28 (7.1)[b]
4	8	8/8 (100.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)

[a] significantly different from group 1 ($P < 0.05$ for the Wilcoxon rank sum test)

[b] significantly different from group 1 ($P < 0.01$ for the Wilcoxon rank sum test)

Table II. Incidence of neoplasm on tongue in F344 rats

Group	No. of rats examined	No. of rats (%)			
		Normal	Hyperplasia	Dysplasia	Invasive SCC
1	32	4/32 (12.5)	5/32 (15.6)	9/32 (28.1)	14/32 (43.8)
2	30	1/30 (3.3)	12/30 (40.0)	8/30 (26.7)	9/30 (30.0)
3	28	2/28 (7.1)	7/28 (25.0)	12/28 (42.9)	7/28 (25.0)
4	8	8/8 (100.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)

ILLUSTRATIONS

Fig. 1. Experimental protocol. ▨ 4-NQO, 20 ppm in drinking water; ☐ basal diet and tap water; ⇧ injection of placebo microspheres; ⬆ injection of atRA-loaded microspheres, 50 mg atRA/kg.

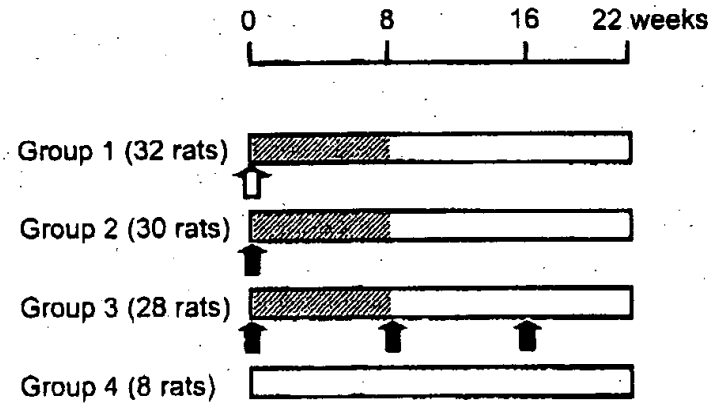
Fig. 2. (a) Hard palate and (b) tongue of 4-NQO treated rats after staining with Lugol's dye solution. (yellowish areas were considered as suspicious regions of dysplasia and carcinoma, and transverse cutting of these tissues were performed through the regions indicated by arrows).

Fig. 3. The concentration profile of atRA in plasma after injecting atRA-loaded microspheres subcutaneously at 50 mg atRA/kg.

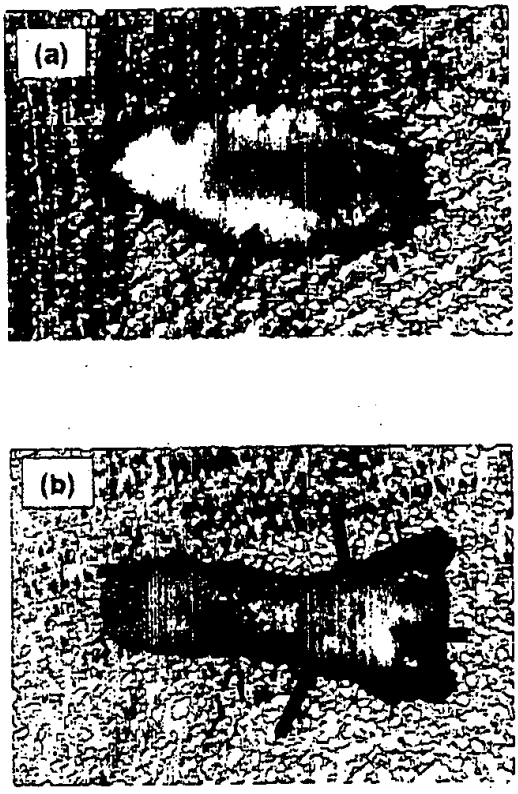
Fig. 4. Body weight changes: (a) the group 1, (b) the group 2, and (c) the group 3. ● represents the mean body weight of rats and ▼ represents the body weight changes of rats with projected tumor.

Fig. 5. (a) Projected tumors on tongue mucosa and (b) sizes of the projected tumors of each tongue. (c) Hard palate in which invasive SCC is developed.

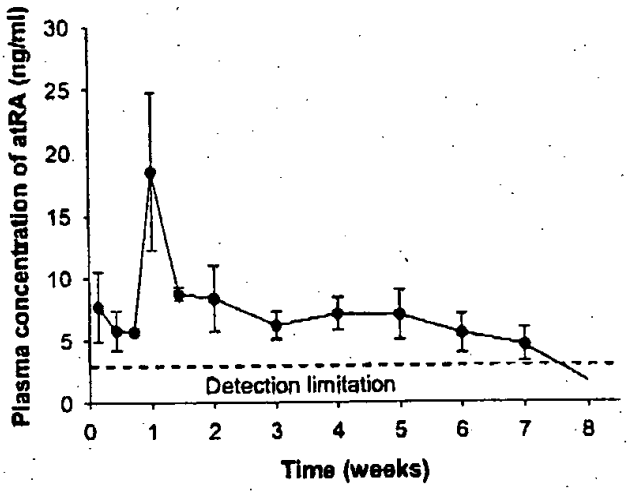
Fig. 6. Representative histology appearance of hard palate epithelium of rat (H&E staining): (a) normal, (b) hyperplasia, (c) dysplasia and (d) invasive SCC.



Yongdoo Choi, Fig. 1

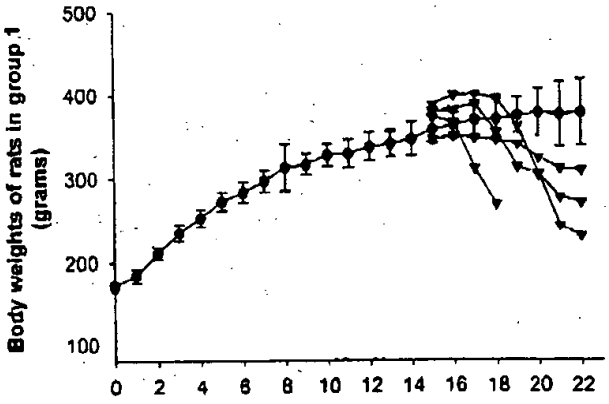


Yongdoo Choi, Fig. 2

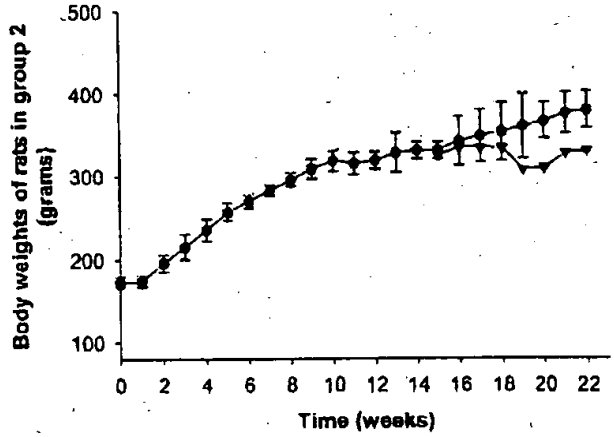


Yongdoo Choi, Fig. 3

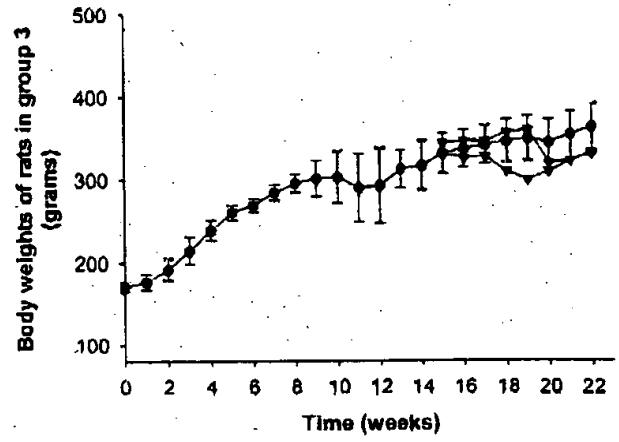
(a)



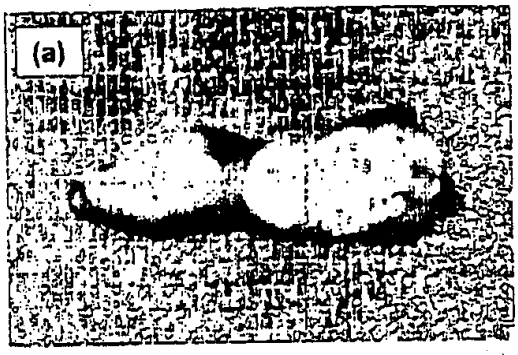
(b)



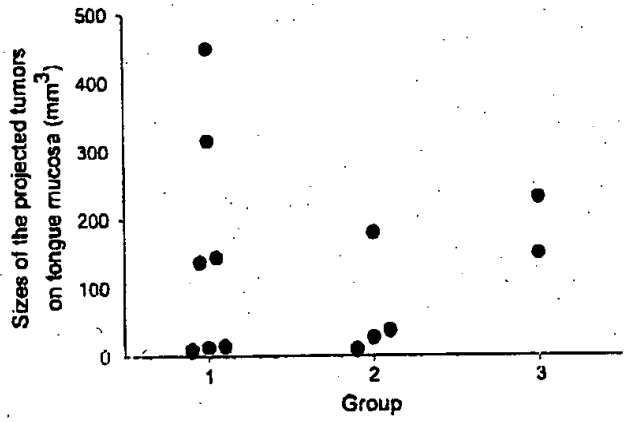
(c)



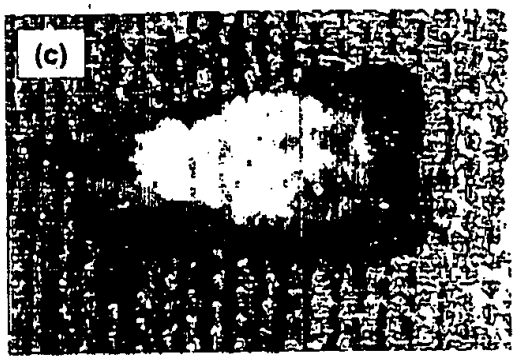
Yongdoo Choi, Fig. 4



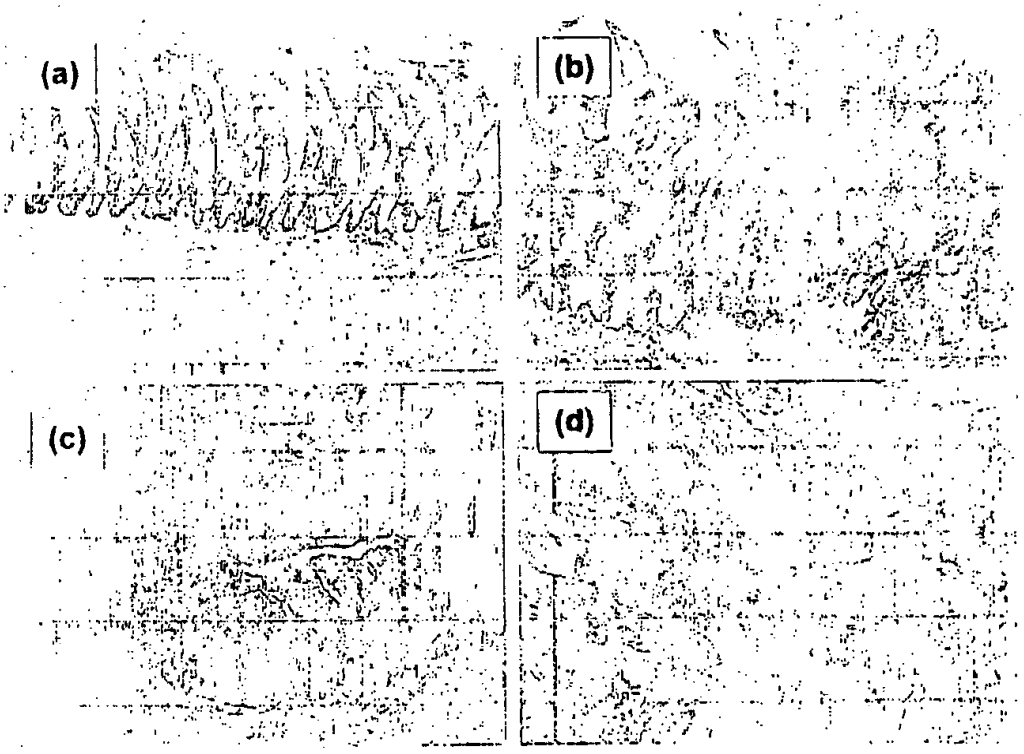
(b)



Yongdoo Choi, Fig. 5



Yongdoo Choi, Fig. 5 (continued)



Choi Y. et al., Fig. 6